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14. ABSTRACT

The purpose of this work was to determine how changes in laminin chains associated with senescence affected prostate cancer progression. In order to determine the effects of alterations to the laminin component of the tumor microenvironment, I overexpressed the laminin alpha 4, beta 2, or alpha 4 and beta 2 chains in a human prostate cancer cell line. I demonstrated that these lines secreted the specific laminin chains and incorporated them into the extracellular matrix. Stable expression of the laminin alpha 4 chain resulted in increased in vitro migration and increased in vivo angiogenesis and tumorigenicity of those cells compared to empty vector control cells. High expression of both the laminin alpha 4 and beta 2 chains decreased in vitro proliferation and migration and decreased in vivo tumorigenicity compared to control cells. Stable expression of the laminin beta 2 chain alone had a tumor promoting function as opposed to the tumor suppressive role seen with high protein levels of the laminin alpha 4 and beta 2 chains together. These data support the concept that since senescent cells secrete both tumor promoting and tumor inhibiting factors, it is the interaction of these factors that influences cell behavior. This dichotomy also points to the importance of understanding

15. SUBJECT TERMS

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INTRODUCTION:

Age-associated epithelial cancers, such as breast and prostate, contribute significantly to the mortality of the elderly. One mechanism by which the body defends itself against epithelial cancers is to halt replication of damaged cells by senescence, in which cells are replicatively arrested but metabolically active. We have shown that senescent prostate cancer cells specifically alter the laminin component of their extracellular matrix (ECM) (Appendix C). Laminins are ECM proteins important to both the structure and function of the microenvironment. Our cell line model of a senescent prostate cancer cell, the M12mac25 cells, displayed increased expression of the laminin α4 and β2 chains. The purpose of this study was to determine how changes in laminin chains associated with senescence affected prostate cancer progression.

BODY:

In order to determine the effects of alterations to the laminin component of the tumor microenvironment, I overexpressed the laminin α4, β2, or α4β2 chains in a human prostate cancer cell line. I demonstrated that these lines secreted the specific laminin chains and incorporated them into the extracellular matrix. Stable expression of the laminin α4 chain resulted in increased *in vitro* migration and increased *in vivo* angiogenesis and tumorigenicity of those cells compared to empty vector control cells. High expression of both the laminin α4 and β2 chains decreased *in vitro* proliferation and migration and decreased *in vivo* tumorigenicity compared to control cells. Stable expression of the laminin β2 chain alone had a tumor promoting function as opposed to the tumor suppressive role seen with high protein levels of the laminin α4 and β2 chains together. These data support the concept that since senescent cells secrete both tumor promoting and tumor inhibiting factors, it is the interaction of these factors that influences cellular behavior. This dichotomy also points to the importance of understanding what factors are secreted by senescent cancer cells and how those factors influence regression or progression of the remaining tumor (see Appendix B for submitted manuscript of research).

This training grant was funded for 2 years; however, since I completed my degree in December 2007, only one year of research was funded and completed for this grant. I was able to complete research for Aim 1: Demonstrate that alterations in laminin composition modulate proliferation and tumorigenicity of prostate epithelial cells *in vitro* and *in vivo*, but did not complete research for Aim 2: Demonstrate that senescence-induced changes in laminins alter

adhesion, proliferation, and transformation of surrounding epithelial cells, or Aim 3: Determine if altered integrin-laminin interactions are a mechanism by which senescent epithelial cells affect cell behavior of normal, initiated, and tumorigenic epithelial cells. Research for Aim 2 has not begun. A preliminary examination of Aim 3 suggests that alterations in integrin β_1 protein expression may be involved in the changes in cellular behavior observed when laminin chain expression is modified, but none of the functional studies mentioned in the grant have been performed.

KEY RESEARCH ACCOMPLISHMENTS:

1. Creation of prostate cancer cell lines overexpressing various laminin chains (α4, β2 or both α4β2).
2. Demonstrated secretion and deposition of these chains into the extracellular matrix; this had not been accomplished for any laminin chains in prostate cell lines before.
3. The novel finding that high expression of the laminin α4 and β2 chains together in prostate cancer cells led to a 3D deposition pattern for laminins as well as other matrix proteins, such as fibronectin. Previously, 3D deposition of laminins was reported only in co-cultures of epithelial cells and fibroblasts.
4. Stable expression of the laminin α4 chain resulted in increased *in vitro* migration and increased *in vivo* angiogenesis and tumorigenicity of those cells compared to empty vector control cells.
5. High expression of both the laminin α4 and β2 chains decreased *in vitro* proliferation and migration and decreased *in vivo* tumorigenicity compared to control cells.
6. Studies of the laminin β2 chain alone have not been described previously. I found that stable expression of the laminin β2 chain alone in prostate cancer cells increased *in vitro* proliferation and migration and had a tumor promoting function (without increased angiogenesis) *in vivo* as opposed to the tumor suppressive role seen with high protein levels of the laminin α4 and β2 chains together.

REPORTABLE OUTCOMES:

1. Successful development of prostate cancer cell lines that overexpress, secrete, and deposit various laminin chains; these lines can be used in future research examining importance of matrix components in progression of prostate cancer.

2. Completion of doctoral program in Molecular and Cellular Biology at the University of Washington, December 2007. **Note that no further laboratory research was performed for this proposed project after December 2007 since funding was terminated at that time due to completion of my degree requirements.**
3. Presented work at the American Association of Cancer Research's annual meeting in April 2008 (Appendix A).
4. Publication of peer-reviewed minireview regarding changes in extracellular matrix components with aging and cancer (Appendix B).
5. Publication of work (Appendix C).
6. Mentor received NIH funding for a grant proposal expanding upon this work, which will further examine the importance of changes in laminins during senescence and prostate cancer progression.

CONCLUSION:

Expression of either the laminin $\alpha 4$ or $\beta 2$ chains alone resulted in increased *in vitro* migration and increased *in vivo* tumorigenicity, while high expression of both chains resulted in decreased *in vitro* proliferation and migration and decreased *in vivo* tumorigenicity. Expression of the laminin $\alpha 4$ chain also resulted in significantly greater angiogenesis of the laminin $\alpha 4$ tumors compared to the other tumors ($p<0.05$) (Appendix B). These data support the concept that since senescent cells secrete both tumor promoting and tumor inhibiting factors, it is the interaction of these factors that influences cellular behavior. This dichotomy also points to the importance of understanding what factors are secreted by senescent cancer cells and how those factors influence regression or progression of the remaining tumor.

It has become increasingly clear that the microenvironment of cells has an important influence on cell behavior. In order to better understand the process of cancer, we must study not only how the cells themselves are altered, due to changes in expression of oncogenes and tumor suppressors, but also how the microenvironment is transformed. Senescent cells alter their matrix and in the process affect neighboring cells, both positively and negatively. By examining the ECM of senescent epithelial cells, we will gain insight into how these cells are transforming their surroundings and the consequences of these alterations on neighboring epithelial cells' potential tumorigenicity. Specifically, the role of laminins as a component of ECM in the prostate has been understudied. Recent research, including the data generated during this

training grant period, points to an increasingly important role of laminins in cancer behavior. I have found this to be especially relevant to the senescence process. Since many chemotherapeutic agents act through induction of a senescence process, this work lays the foundation for future studies that will further elucidate the senescent mechanisms of chemotherapy and its impact on progression of prostate cancer. In addition, human integrin blocking antibodies, growth-factor receptor blocking antibodies, and angiogenesis inhibitors are currently in clinical trials for other cancers.[1-5] Since alterations in laminins determine, in part, the role of integrins and growth factor receptors in prostate cancer progression and angiogenesis, the results of these future studies may lead to potential new therapies for prostate cancer.

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3. Sprenger CC, Drivdahl RH, Woodke LB, Eyman D, Reed MJ, Carter WG, and Plymate SR (2008) Senescence-induced alterations of laminin chain expression modulate tumorigenicity of prostate cancer cells. *Neoplasia* **10**: 1350-1361.

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Cynthia Sprenger, PhD

APPENDIX A
Abstract for AACR meeting (April 2008)

Senescence-induced alterations of laminin chain expression modulate progression of prostate cancer

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Introduction: Age-associated epithelial cancers, such as breast and prostate, contribute significantly to the mortality of the elderly. One mechanism by which the body defends itself against epithelial cancers is to halt replication of damaged cells by senescence, in which cells are replicatively arrested but metabolically active. We have shown that senescent prostate cancer cells specifically alter the laminin component of their extracellular matrix (ECM). Laminins (LM) are ECM proteins important to both the structure and function of the microenvironment. Our cell line model of a senescent prostate cancer cell, the M12mac25 cells, displayed increased expression of the laminin α4 and β2 chains.

Purpose: The purpose of this study was to determine how changes in LM chains associated with senescence affected prostate cancer progression.

Methods: In order to determine the effects of alterations to the laminin component of the tumor microenvironment, we stably transfected the M12 cell line with cDNAs for the LMα4, β2, or α4β2 chains. Using Western immunoblots and immunofluorescent staining, we demonstrated that these lines secreted the specific LM chains and incorporated them into the ECM. We then measured *in vitro* proliferation (MTS assay) and migration (wounding assay) of these cells and injected $1x10^6$ cells from each construct subcutaneously into nude mice to measure *in vivo* tumorigenicity. Immunohistochemistry against mouse endothelial cell antigen (MECA) was used to assess angiogenesis of the tumors.

Results: Stable expression of the LMα4 chain in the M12 prostate cancer cell line resulted in increased *in vitro* migration and increased *in vivo* angiogenesis and tumorigenicity of those cells compared to the M12 empty vector (M12pc) cells. High expression of both the LMα4 and β2 chains decreased *in vitro* proliferation and migration and decreased *in vivo* tumorigenicity

compared to M12pc cells. Stable expression of the LMβ2 chain alone had a tumor promoting function as opposed to the tumor suppressive role seen with high protein levels of the LMα4 and β2 chains together.

Summary: Expression of either the LMα4 or β2 chains alone resulted in increased *in vitro* migration and increased *in vivo* tumorigenicity, while high expression of both chains resulted in decreased *in vitro* proliferation and migration and decreased *in vivo* tumorigenicity. Expression of the LMα4 chain also resulted in significantly greater MECA staining in the LMα4 tumors compared to the other tumors ($p<0.05$). These data support the concept that since senescent cells secrete both tumor promoting and tumor inhibiting factors, it is the interaction of these factors that influence cellular behavior. This dichotomy also points to the importance of understanding what factors are secreted by senescent cancer cells and how those factors influence regression or progression of the remaining tumor.

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APPENDIX B
Peer-reviewed Minireview

Minireview

Extracellular influences on tumour angiogenesis in the aged host

CC Sprenger[1,2], SR Plymate[2,3] and MJ Reed[*,2]

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Whether tumours are epithelial or non-epithelial in origin, it is generally accepted that once they reach a certain size all solid tumours are dependent upon a vascular supply to provide nutrients. Accordingly, there is great interest in how the extracellular environment enhances or inhibits vascular growth. In this minireview, we will examine key extracellular components, their changes with ageing, and discuss how these alterations may influence the subsequent development of tumour vasculature in the aged host. Because of the tight correlation between advanced age and development of prostate cancer, we will use prostate cancer as the model throughout this review.

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Keywords: prostate cancer; microenvironment; senescence; angiogenesis; growth factors; matrix metalloproteinases

During an organism's lifespan, almost every aspect of its phenotype will undergo alterations, including the components of the extracellular environment. It is increasingly evident that there is a dynamic interaction between the molecules of the extracellular space and the surrounding cells. The architecture of the extracellular space is important for maintaining proper cellular function; loss of tissue architecture is a defining characteristic of epithelial cancers. A microenvironment that provides the correct cues can serve as a powerful tumour suppressor and can even revert cells containing preneoplastic as well as oncogenic mutations back to a normal phenotype (Bissell and Radisky, 2001; Campisi, 2005; Nelson and Bissell, 2006). The processes of living and ageing, however, continually alter the composition, and thus the architecture, of the extracellular space.

Traditionally, interactions between tumour cells and various growth factors have been the focus in cancer. However, there is increasing interest in the roles of other proteins in the extracellular environment on tumour progression. The term 'microenvironment' now includes the extracellular matrix (collagens, laminins, matricellular proteins) and soluble factors (hormones, cytokines, growth factors, enzymes) that are released by resident and circulating cells or secreted by other organs.

All non-circulating cells are physically linked to the extracellular space via cell membrane receptors such as integrins and syndecans. Signalling through these receptors influences changes to the cell's cytoskeleton network, which is connected to the nuclear matrix and chromatin. Alterations in the cytoskeleton modify gene expression, which in turn leads to changes in the chemical and protein composition of the microenvironment (Nelson and Bissell, 2006). In epithelial cancers, transformed epithelial cells, reactive stroma, recruited blood vessels, and infiltrating macrophages, lymphocytes, and leukocytes also contribute to the microenvironment (Nelson and Bissell, 2006; Tan and Coussens, 2007). In this review, we will use prostate cancer as the model and focus on the potential roles of extracellular matrix proteins and soluble factors during tumour angiogenesis in the aged host (Figure 1).

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EPITHELIAL CANCERS

Age-associated epithelial cancers, such as breast and prostate cancer, contribute significantly to mortality in the elderly. One possible mechanism by which the body defends itself against epithelial cancers is to halt replication of damaged cells by senescence, in which the cells are replicatively arrested but still metabolically active. Since somatic mutations are believed to accumulate throughout life, senescence is important in preventing the formation of epithelial tumours in the young. Accumulation of mutations alone, however, is not sufficient to cause cancer. Currently, one view is that these 'initiated' cells require a permissive microenvironment in which to progress (Campisi, 2005; Nelson and Bissell, 2006). The accruement of senescent cells may provide such an environment due to factors secreted by these cells that compromise tissue structure and function. Once a cell has entered senescence, its transcriptome is altered such that genes associated with angiogenesis are activated. Inflammatory cytokines (IL-8), growth factors (TGF-β, EGF), matrix metalloproteinases (MMPs), and extracellular matrix proteins (laminins, collagens, fibronectin) are among the genes upregulated by senescent cells (Zhang *et al*, 2003; Campisi, 2005; Bavik *et al*, 2006). This alteration in expressed genes affects not only the senescent cell itself, but the cells surrounding it as well. Senescent fibroblasts that were co-cultured with breast or prostate epithelial cells increased the proliferation and tumorigenicity of those epithelial cells, both *in vitro* and *in vivo* (Campisi, 2005; Bavik *et al*, 2006). Senescence, then, inhibits cancer formation early on but over time the build up of senescent cells alters the microenvironment to one that can promote the initiation of epithelial cancers.

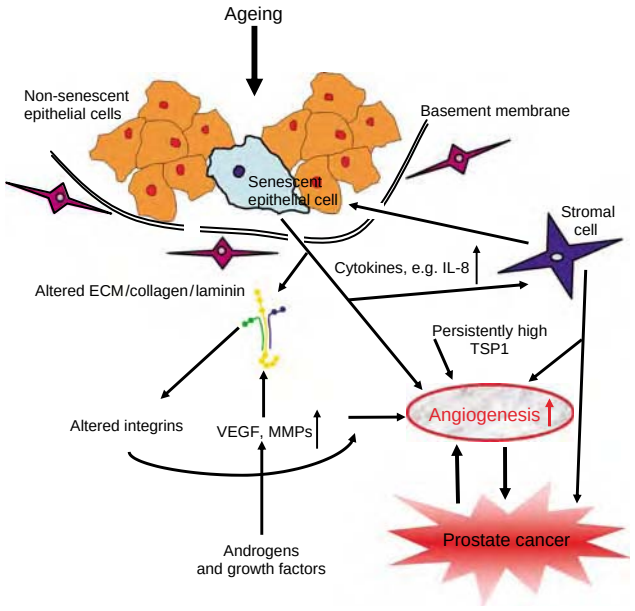

Figure 1 Effects of the aging microenvironment on angiogenesis of prostate tumours. The presence of senescent cells increases with age. These cells alter their expression of ECM proteins, which in turns modifies the composition of the microenvironment. Although the stromal cell has not been shown to senesce in the prostate, the cytokines produced by senescent epithelium influence stromal cell function and secretions. Local increases in hormones, growth factors (such as IGF-1), and matricellular proteins such as thrombospondin (TSP1) further alter the microenvironment. The aged prostate microenvironment, therefore, contains many components that are pro-angiogenic, thus supporting the growth of transformed epithelial cells and enhancing angiogenesis of the primary tumour in the aged host.

Clinical observations suggest that while ageing confers the greatest risk of developing cancer, once initiated, histologically similar tumours behave less aggressively in the aged individual (Ershler, 1986). This premise was further supported by animal studies in which young and aged mice received identical inocula of tumour cells and were subsequently monitored for tumour growth and aggressiveness (Kreisle *et al*, 1990; Pili *et al*, 1994). Proposed mechanisms for differences in tumour behaviour in young *vs* aged hosts have focused on age-related deficits in immune-mediated responses that directly and indirectly promote tumour growth (such as a lack of inflammatory cells and their associated cytokines), increases in apoptosis, and decreases in pathological angiogenesis (Ershler, 1986; Kreisle *et al*, 1990; Pili *et al*, 1994). The aged microenvironment, it has been argued, is less permissive to pathological angiogenesis and tumour growth than the milieu of tissues found in the young. Such an alteration in tissue architecture has been thought to be an adaptive response to the greater risk of cancer conferred by senescence and environmentally induced changes in the epithelial and stromal cells (Campisi, 2005). More recently, however, we have shown that prostate epithelial tumours can provide a microenvironment that allows certain tumours to grow equally well in aged and young mice (Reed *et al*, 2007).

The aged microenvironment, likewise, affects the metastasis of the primary tumour. Recent data from Kaplan *et al* (2006) suggest that tumour metastases are determined by preparation of a bone marrow-derived 'metastatic niche' prior to the arrival of cancer cells (Kaplan *et al*, 2006). When aged marrow was used to replace the marrow in young irradiated mice, tumour metastases decreased. Conversely, when marrow from young donors was used to replace marrow in older irradiated mice, metastases increased. Langley and Fidler (2007) further reviewed the myriad of factors involved in metastases, including the angiogenic component, and concluded that interactions of the tumour cells with the host homoeostatic mechanisms are highly variable and complex (Langley and Fidler, 2007). Therefore, the reasons behind an apparent decrease in metastases in the aged host *vs* young host are many fold and likely vary with the type of cancer. In this minireview, we will focus on how age-induced modifications of the microenvironment influence angiogenesis of the primary tumours.

EXTRACELLULAR MATRIX

The specific effects of age on the extracellular matrix have not been well delineated within the tumour microenvironment. However, studies of age-related alterations of the matrix in other tissues, and of changes in tumour matrix in non-aged hosts, provide a basis for discussion of collagen and laminin, the best studied of these proteins.

Collagen I is a heterotrimeric, fibrillar protein that is the major structural extracellular protein in most tissues (Chung *et al*, 2005). Collagen I has been the most extensively examined collagen in aged hosts and the consensus is that ageing confers a progressive decrease in collagen I synthesis at the same time there is an increase in collagen I degradation. There are important exceptions to this premise, such as the increased collagen I deposition that is often noted in aged hearts (Gazoti Debessa *et al*, 2001). Although the cardiac changes are primarily a response to injury or hypertension, and not ageing *per se*, the observations with respect to collagen I underscore the need to use the term 'deregulation' to describe many of the changes in the matrix in aged organs.

Studies examining mechanisms of decreased collagen I content in aged tissues have noted that lower levels of fibrogenic growth factors, such as transforming growth factor-β (Ashcroft *et al*, 1997), contribute to less collagen I synthesis and subsequent scarring. At the same time, elevated matrix metalloproteinase activity mediates increased collagen I degradation. Whether the latter results from an increase in collagenase and other MMPs or a decrease in tissue inhibitors of MMPs is still a matter of debate, but the end result is a looser, less organized collagen network (Hornebeck *et al*, 2002). Whereas some have suggested that a less dense collagen matrix facilitates vascular in-growth (Reed *et al*, 2005), studies of angiogenesis in most organs have demonstrated decreased capillary density with age (Rivard *et al*, 1999). Alterations in collagen I that have functional consequences include age-related deficits in integrin-collagen binding that result in less robust cell adhesion and migration (Reed *et al*, 2001), which could contribute to delayed tissue repair.

Although diminished collagen I content results in less scarring and fibrosis in most aged tissues (with the exception of the heart as noted above), the implications for tumour angiogenesis and growth are largely a matter of conjecture and depend on the tumour cell type. For example, we found that the amount of collagen I in melanomas from aged mice was decreased compared to levels found in prostate tumours from aged mice; likewise, we found decreased vessel density in these melanomas compared to the prostate tumours (Reed *et al*, 2007). The therapeutic implications are especially of interest in the treatment of cancers that produce large quantities of collagen such as prostate. One could surmise that if a cancer cell can be modified to secrete less collagen, there will be decreased support for vascular in-growth and subsequent tumour progression.

While collagen I is the best-studied extracellular matrix protein in non-tumour aged tissues, the examination of laminins in tumour angiogenesis has been restricted to non-aged hosts. Laminins (LM) are large matrix glycoproteins composed of highly homologous α, β, and γ chains and are the main constituent of basal membranes (a special matrix that separates different cell types from one another, such as endothelial or epithelial cells from

the surrounding stroma). Laminins are crucial components of the tissue architecture, as well as modulators of cell behaviour (Patarroyo *et al*, 2002). The laminin $\alpha 4$ chain is expressed by most endothelial cells throughout the body and plays an important role in post-developmental angiogenesis associated with inflammation and tumours (Zhou *et al*, 2004). LM411 ($\alpha 4\beta 1\gamma 1$) (formerly known as laminin-8) facilitates endothelial proliferation and protects endothelial cells from apoptosis (DeHahn *et al*, 2004). Sprouting and tumour blood vessels express LM411, whereas normal blood vessel maturation and loss of malignant characteristics are associated with conversion to LM421 ($\alpha 4\beta 2\gamma 1$) (formerly known as laminin-9) (Zhou *et al*, 2004). LM411 facilitates tumour progression, but the presence of LM421 may be equally important in preventing metastases, as *lama4*$^{-/-}$ mice exhibit uncontrolled blood vessel proliferation following injury and have increased tumour growth and metastasis (DeHahn *et al*, 2004; Zhou *et al*, 2004).

Senescent stromal cells highly secrete laminins. Accordingly, laminins have been postulated to influence the cancer phenotype of breast and prostate epithelium. Recent studies have shown that senescent prostate epithelial cells found in regions of benign prostatic hyperplasia as well as senescent prostate fibroblasts have increased expression of the laminin $\alpha 4$ and $\beta 1$ chains (Luo *et al*, 2002; Bavik *et al*, 2006). Fujita *et al* (2005) also demonstrated a switch from LM421 to LM411 expression in breast tumour vasculature, implying that increased expression of the LM411 chains may be associated with progression of some epithelial cancers (Fujita *et al*, 2005). This group recently reported regression of glioblastoma tumours in mice following administration of LM411 inhibitors (Fujita *et al*, 2006). It would be of interest to examine if a similar outcome occurred in mouse models of breast or prostate cancer since aberrant expression of LM411 by senescent and epithelial tumour cells appears to influence the angiogenic potential of adjacent endothelial cells. Thus, in the aged host, accumulation of senescent cells may facilitate epithelial tumour growth, in part, via increased expression of laminins associated with tumour angiogenesis.

MATRICELLULAR PROTEINS

The term matricellular refers to proteins of the extracellular space that regulate cell function without providing significant structural support. Although the size of the family of molecules designated as 'matricellular' continues to grow, only a few member proteins have been examined in aged hosts. Key molecules investigated in ageing and in tumour biology include thrombospondin 1 (TSP1) and secreted protein acidic and rich in cystein (SPARC).

Thrombospondin 1 is a large heterotrimer whose expression increases in most aged cells and tissues (Naumov *et al*, 2006). The negative effects of TSP1 on endothelial cell function have resulted in great interest in this protein in tumour angiogenesis and progression. In many cancers, TSP1's presence is associated with a non-angiogenic phenotype and tumour regression; the absence of TSP1 expression is correlated with an angiogenic switch and metastases (Naumov *et al*, 2006). Thrombospondin 1 inhibits angiogenesis by blocking growth factor-mediated angiogenic functions such as proliferation and migration as well as by enhancing apoptosis in activated endothelial cells (Colombel *et al*, 2005).

In the prostate, androgens repress the transcription of TSP1. However, it is now understood that the clinical implications of TSP1 expression on tumour vascularity and growth depend on the duration of exposure. Androgen withdrawal initially leads to increases in TSP1 and vessel regression; however, with continued exposure prostate cancer angiogenesis and growth continue despite persistently high levels of TSP1 (Colombel *et al*, 2005). Similar results have been reported in breast cancers: persistently high levels of TSP1 in the tumour stroma ultimately result in disease progression, an effect that may result from increased expression of VEGF (Fontana *et al*, 2005). These conflicting effects have dampened enthusiasm for the use of fragments of TSP1 in clinical intervention studies.

Secreted protein acidic and rich in cystein is a secreted glycoprotein that is highly expressed in injured and inflamed tissues. Accordingly, high levels of SPARC are found in many aged organs and in numerous cancers (Framson and Sage, 2004). Intact SPARC inhibits the angiogenic response by impairing proper collagen alignment and blocking pro-angiogenic growth factors (Kupprion *et al*, 1998). At the same time it has been reported that cleaved SPARC might facilitate vessel growth by enhancing endothelial cell proliferation (Sage *et al*, 2003). In aged tissues, the complexities surrounding the role of SPARC in the angiogenic response are obviated by age-related deficits in the levels of growth factors and other pro-angiogenic mediators (Reed *et al*, 2005). Nevertheless, the relative ease by which *in vivo* SPARC expression can be manipulated has resulted in continued enthusiasm for its therapeutic potential in highly vascularized tumours (Elola *et al*, 2007).

ANDROGENS

Although serum androgen levels decrease with age, levels of active androgen in the prostate, dihydrotestosterone, do not decrease due to increased activity of the 5α-reductase enzyme, which converts testosterone (T) to dihydrotestosterone (Bonnet *et al*, 1993). In malignant prostate epithelium androgens can stimulate angiogenesis (Colombel *et al*, 2005). Following androgen withdrawal in androgen-dependent tumours, there is a decrease in angiogenesis associated with tumour regression. However, there is invariably a return of tumour that is castration resistant. These tumours are commonly referred to as androgen-independent (AI), although castration-resistant may be a more appropriate term since they still contain significant levels of T and dihydrotestosterone (Mostaghel *et al*, 2007). In castration-resistant tumours, there is an increase in angiogenesis that is associated with an increase in MMP-9 (London *et al*, 2003). These studies indicate that tumour angiogenesis in prostate cancer is associated first with androgens, then with an increase in matrix remodelling proteases. Further, these data imply that anti-angiogenic drugs should be of potential therapeutic benefit. However, no definitive clinical trials have been published, which may reflect a unique ability of prostate cancer to bypass the usual age-associated inhibition of angiogenesis.

GROWTH FACTORS

Although many growth factors regulate the angiogenic response in the tumour microenvironment, we will focus our discussion on vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1). These two traditional growth factors directly facilitate endothelial cell functions that promote blood vessel formation and have been examined in prostate cancer progression. Other mediators, such as IL-8 and associated inflammatory cytokines that modulate endothelial cell behaviour, will not be discussed due to their intricate relationship with the immune system of the host during tumour initiation and growth.

It is generally accepted that ageing compromises the ability of cells to produce angiogenic growth factors, including VEGF (Rivard *et al*, 1999). Vascular endothelial growth factor is the most potent of the numerous mediators that induce endothelial cell functions and facilitate new vessel formation. The primary stimulus for VEGF synthesis is hypoxia. However, the response to low oxygen tension is blunted in aged tissues as a result of defects in hypoxia-inducible factor 1 (HIF-1), the transcription factor responsible for VEGF synthesis (Rivard *et al*, 2000). Although one

would predict that decreased VEGF expression would confer an element of protection against tumour vascularisation and subsequent growth in aged hosts, the clinical data supporting this premise are lacking. In prostate cancer, while VEGF levels are not predictive of positive biopsy results (Peyromaure *et al*, 2005) higher plasma levels of VEGF are associated with metastases and a poorer prognosis (Duque *et al*, 1999). Using a transgenic mouse model of prostate cancer, Isayeva *et al* (2007) demonstrated that inhibitors of the VEGF2 receptor delayed tumour progression only when administered in the early stages of prostate cancer, before a significant rise in VEGF levels was observed. This same inhibitor was ineffective if administered during the later stages of prostate cancer, when VEGF levels were high (Isayeva *et al*, 2007). Thus, the minimal effects on tumour progression in clinical trials of angiogenesis inhibitors may be due to the advanced stage of prostate cancer being targeted. It is, therefore, likely that the decrease in VEGF seen in many tissues with ageing does not inhibit the development of prostate cancers in aged men. Administration of angiogenesis inhibitors may be more effective if given earlier in the course of prostate cancer progression, prior to a rise in VEGF levels, or in conjunction with other therapeutic interventions such as androgen ablation.

Insulin-like growth factor-1 is a potent stimulator of cellular proliferation and survival as well as tumour growth. While serum levels of IGF decrease with age, within the aged population those individuals with the highest levels of serum IGF-1 have the greatest risk of developing epithelial cancers such as prostate cancer (Kaplan *et al*, 1999). During progression of prostate cancer, local levels of both IGF-1 and its receptor (IGF-1R) increase (Kaplan *et al*, 1999). Like many growth factors, IGF-1 has the potential to reverse the age-associated decline in endothelial cell function (Thum *et al*, 2007). Moreover, IGF-1 upregulates the expression of modulators of endothelial cell function such as VEGF, MT1-MMP, and MMP2; this regulation requires signalling through the IGF-1 receptor via both the PI3 kinase and MAP kinase pathways (Miele *et al*, 2000; Grzmil *et al*, 2004; Zhang *et al*, 2004). Goel *et al* (2004) reported that interactions between the IGF-1R and β_1 integrins also activated signalling through both the PI3 kinase and MAP kinase pathways, which resulted in enhanced prostate tumour cell migration on and invasion through the extracellular matrix (Goel *et al*, 2004). Although tumour angiogenesis has been associated with increased expression of β_1 integrins and increased signalling through the PI3K pathway (Stupack and Cheresh, 2002), the direct effect of IGF-1R-integrin β_1 interactions on vessel growth in cancers has not been studied. Accordingly it is implied, but not proven, that increased levels of IGF-1 in the aged prostate could promote endothelial cell function thereby resulting in similar levels of vascularisation, and primary tumour growth, in young and old hosts.

MATRIX METALLOPROTEINASES

The extracellular environment contains numerous classes of enzymes that regulate the controlled degradation of matrix proteins. Many of these proteases also modulate other cellular functions, either directly by interacting with receptors at the cell surface or indirectly by activation of latent molecules in the extracellular milieu. Within the context of tumour progression and angiogenesis, the matrix metalloproteinases (MMPs) are the most widely studied class of molecules. Although it is a widely held belief that tissue levels of MMPs increase with ageing, more recent studies indicate that changes in MMP activity in most aged organs reflect a deregulation rather than pervasive increases (Reed *et al*, 2000). Accordingly, some aged tissues show decreased matrix turnover at the same time others demonstrate increased MMP activity.

During cancer progression, organ and tumour cell-specific changes in MMPs, rather than host age, determine the influence of matrix degradative enzymes on subsequent tumour growth. The lack of a specific and pervasive age effect on MMP levels is important clinically as it is generally accepted that the ability of solid tumours to express gelatinases is positively correlated with their invasive potential and subsequent poor clinical outcomes (Mancini and Di Battista, 2006). Prostate tumours, in particular, express increasing amounts of MMP2 and MMP9 as they progress to higher-grade tumours and greater degrees of metastatic potential (Wood *et al*, 1997). The influence of MMPs on tumour propagation results from both direct and indirect mechanisms (Mancini and Di Battista, 2006). Direct effects, via degradation of the matrix, result in a more permissive environment for cell migration and invasion. The subsequent facilitation of the angiogenic response results in a greater blood supply to the tumour. Indirect effects of MMP activity include activation of other pro-MMPs, cleavage of regulatory precursor molecules at the cell surface, and induction of nascent chemokines and growth factors that require enzymatic activation.

We have shown in aged animals that angiogenesis and tumour growth are inhibited in some solid tumours, such as melanomas, but in prostate cancer equivalent angiogenesis and tumour growth occurred in both young and old animals. Moreover, the prostate tumours had high levels of gelatinase (MMP2 and MMP9) expression and activity (Reed *et al*, 2007). The relationship between a well-formed matrix and MMP expression is expected as extracellular matrix proteins regulate, in part, the production of the enzymes responsible for their turnover and degradation (Phillips and Bonassar, 2005). Once tumour cells express MMPs, they can induce MMP secretion from their associated stromal cells thereby further amplifying their potency (Stuelten *et al*, 2005). MMP activity also has been shown to be a key component of VEGF-induced angiogenesis in tumours (Bergers *et al*, 2000), reflecting another pathway by which MMPs interact with components of the ECM to facilitate vessel formation and tumour growth. Although the modulation of MMPs has resulted in minimal effects in the therapeutic arena, it is notable that these studies employed general MMP inhibitors. The development of more specific and potent MMP inhibitors, in conjunction with other interventions, may result in improved clinical efficacy.

CONCLUSION

Location, location, location: body-wide levels of factors associated with angiogenesis may decrease with ageing, but their level of expression can increase locally. The prostate provides a unique model for this paradigm. For example, while serum levels of IGF-1 decrease with age, prostatic levels increase during prostate cancer progression. This local increase in IGF-1 leads to an upregulation in factors, such as VEGF, MT1-MMP, and MMP2, which modulate endothelial cell function and subsequent angiogenesis. Furthermore, senescent cells are more prevalent with host age and display a transcriptome that parallels angiogenesis. Growth factors, cytokines, MMPs, collagens, laminins, and integrins are all upregulated by senescent cells. Senescent fibroblasts and epithelial cells may subsequently alter the local microenvironment to one that promotes angiogenesis and epithelial tumour growth. Consequently, while angiogenesis is generally impaired in aged tissues, the local microenvironment of primary epithelial tumours in the aged host may be as supportive of angiogenesis as that found in the young.

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Senescence-Induced Alterations of Laminin Chain Expression Modulate Tumorigenicity of Prostate Cancer Cells[1]

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Abstract

Prostate cancer is an age-associated epithelial cancer, and as such, it contributes significantly to the mortality of the elderly. Senescence is one possible mechanism by which the body defends itself against various epithelial cancers. Senescent cells alter the microenvironment, in part, through changes to the extracellular matrix. Laminins (LMs) are extracellular proteins important to both the structure and function of the microenvironment. Overexpression of the senescence-associated gene *mac25* in human prostate cancer cells resulted in increased mRNA levels of the LM α4 and β2 chains compared to empty vector control cells. The purpose of this study was to examine the effects of these senescence-induced LM chains on tumorigenicity of prostate cancer cells. We created stable M12 human prostate cancer lines overexpressing either the LM α4 or β2 chain or both chains. Increased expression of either the LM α4 or β2 chain resulted in increased *in vitro* migration and *in vivo* tumorigenicity of those cells, whereas high expression of both chains led to decreased *in vitro* proliferation and *in vivo* tumorigenicity compared to M12 control cells. This study demonstrates that senescent prostate epithelial cells can alter the microenvironment and that these changes modulate progression of prostate cancer.

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Introduction

Prostate cancer is the most common cancer and the second leading cause of illness and death for men older than 50 years in western countries [1,2]. Possible mechanisms for defense against epithelial cancers, such as prostate, include promotion of apoptosis in which the damaged cell dies or senescence in which the cell ceases to divide but remains metabolically active. An accumulation of mutations, which is believed to occur during the life span of an organism, is not sufficient to cause cancer [3]; instead, these initiated premalignant cells require a permissive microenvironment in which to progress [4,5]. The accrual of senescent cells as an organism ages may provide such an environment owing to secreted factors that compromise tissue structure and function. Studies examining the effects of senescent fibroblasts on the growth of premalignant epithelial cells demonstrated increased growth and tumorigenicity of those epithelial cells [6,7]. Senescence then acts to inhibit cancer formation in a younger organism, but over time, the accumulation of senescent cells alters the microenvironment to one that can promote the growth of epithelial cancers [5–8].

Abbreviations: LM, laminin; LM332, laminin α3β3γ2; LM411, laminin α4β1γ1; LM421, laminin α4β2γ1; LM511, laminin α5β1γ1; LNCaP, human prostate cancer cell line derived from a lymph-node metastasis; *mac25*, senescence-associated gene also known as IGFBP-7/IGFBP-rP1/TAF; M12, human prostate cancer cell line derived from SV-40T–immortalized benign prostate epithelial cells; MECA, mouse endothelial cell antigen; PEC, primary prostate epithelial cells

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Although senescence of fibroblasts has been studied heavily, a paucity of studies on the senescence of epithelial cells has been completed [9–14]. After 30 doublings, cultured primary prostatic epithelial cells stain positive for senescence-associated (SA)-β-galactosidase [9] and exhibit increased protein levels of p16 and mac25 (IGFBP-7/IGFBP-rP1) [10–12]. Staining for SA-β-gal in various prostate tissues demonstrated the presence of senescent epithelial cells primarily in regions of benign prostatic hyperplasia and prostatic intraepithelial neoplasia but rarely in cancer [9,15]. However, reports of chemotherapeutic agents inducing a senescence-like state in cancer cells, including prostate cancer cells, imply that cancer cells are capable of undergoing senescence as well [16–18]. We have demonstrated that transfection of the senescence-associated gene *mac25* into the M12 and LNCaP human prostate cancer cell lines resulted in increased senescence, decreased proliferation, a delay in G_1, and decreased *in vitro* and *in vivo* tumorigenicity [19–21].

Senescent fibroblasts modify the microenvironment [7], but the occurrence of such alterations by senescent cancer cells has not been examined previously. Using cDNA microarrays, we found that senescent M12 and LNCaP prostate cancer cells have increased transcript levels of the laminin (LM) α4 and β2 chains, among other genes (unpublished data). Laminins are a major constituent of the extracellular matrix that link the ECM to cells through various cell surface receptors [22]. They are large, heterotrimeric, cruciform matrix glycoproteins composed of highly homologous α, β, and γ chains; specific LM isoform expression and posttranslational processing can directly influence cellular response to growth factors, intracellular signaling, cell proliferation, susceptibility to apoptosis, and migratory capacity [23]. In various cancers, including breast cancer, increased expression of the LM α4 and β1 chains is associated with increased tumorigenicity and angiogenesis [22,24,25]. In prostate cancer, changes in LM composition within the prostate tumor microenvironment have been associated with the progression of cancer [26]. Studies specifically examining alterations in LM expression during senescence have not been undertaken.

The purpose of this study was to examine the effects of senescence-induced LM chains on the tumorigenicity of prostate cancer cells. We created stable M12 prostate cancer cell lines overexpressing either the LM α4 or β2 chains or both the LM α4 and β2 chains. We demonstrate that overexpression of either the LM α4 or β2 chains increased tumorigenicity of prostate cancer cells, whereas overexpression of both chains decreased tumorigenicity. Our investigation of the effects of senescence on behavior of cancer cells will provide insight into how current prostate cancer therapies influence cancer progression.

Methods

Reagents

Tissue culture media, additives, and antibiotics were purchased from GIBCO (Grand Island, NY). SYBR GREEN PCR Master Mix was from Applied Biosystems (Foster City, CA). The BCA protein assay kit was from Pierce Biological (Rockford, IL). Nitrocellulose and polyacrylamide gel electrophoresis (PAGE) reagents were purchased from BioRad Laboratories (Richmond, CA). Laminin antibodies used in Western immunoblot analyses were obtained from Santa Cruz Biologicals (Santa Cruz, CA), whereas LM and fibronectin antibodies used for immunofluorescent staining were produced at Fred Hutchinson Cancer Research Center (Seattle, WA). Fluorescent-conjugated secondary antibodies were purchased from Invitrogen (Hercules, CA). Horseradish peroxidase–linked anti–rabbit secondary antibody and enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Restriction enzymes were obtained from Promega (Madison, WI). The pcDNA3.1 expression vector was purchased from Invitrogen.

Cell Culture

Primary prostate epithelial cells (PECs) were obtained from Dr. Beatrice Knudsen (Fred Hutchinson Cancer Research Center, Seattle, WA) and grown with keratinocyte growth medium supplemented with epidermal growth factor and bovine pituitary extract. The derivation of the M12 cell line has been previously described [27]. M12 and M12-LM cells were cultured in RPMI 1640 supplemented with 10 ng/ml epidermal growth factor, 0.1 mM dexamethasone, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, fungizone, 50 μg/ml gentamicin, and 5% fetal calf serum at 37°C under 5% CO_2. All of the cells used in these experiments were mycoplasma-free, as determined by the PCR Mycoplasm Test Kit (MD Biosciences, Zurich, Switzerland).

Vector Preparation

The mammalian expression vector pcDNA3.1 (Invitrogen) was used to prepare the LMα4 and β2 chain constructs, which expressed the LM cDNA from the constitutively active cytomegalovirus promoter. The 6.5-kb full-length LMα4 chain (LAMA4) cDNA was obtained from OriGene (Rockville, MD) in their nonselectable vector (pCMV6-XL4). We subcloned the LAMA4 cDNA as an *Eco*RI/*Hin*dIII fragment into pcDNA3.1Neo. The LMβ2 chain (LAMB2) cDNA was generated by polymerase chain reaction (PCR) with *Pfu* DNA polymerase (Promega) using the following primers:

LAMB2
forward: AGACCGTTCACCTCCCCTTATC
reverse: TTCAGTGCATAGGCAGACATGC

A 5.6-kb cDNA fragment containing the full-length coding sequence was ligated into the pcDNA3.1Hygro *Eco*RV site. Orientation of cDNA was determined by restriction digestion.

Transfection

Cell lines overexpressing the LMα4, β2, or both the α4 and β2 chains were produced by liposome-mediated transfection of the M12 human prostate cancer cell line using pFx-5 (Invitrogen) according to the manufacturer's instructions. Transfecting the M12 cells with pcDNA3.1 alone produced control cells. M12α4β2 cells were created sequentially, first by transfection and selection for LMα4 (800 μg/ml G418), then by transfection with the LAMB2 cDNA and selection for LMα4 (800 μg/ml G418) and LMβ2 (100 μg/ml hygromicin). Surviving transfected cells were maintained with either 400 μg/ml G418 (M12α4 cells), 400 μg/ml G418 plus 50 μg/ml hygromicin (M12α4β2 cells), or 50 μg/ml hygromicin (M12β2 cells). Total cell lysates and RNA were isolated and analyzed for expression of various LM chains, including the LMα4 and β2 chains (see the Western Immunoblot Analysis, Immunofluorescent Staining, and Real-time PCR subsections).

Real-time PCR

Total RNA was obtained from monolayer cell cultures using Qiagen RNeasy Plus (Valencia, CA); the optional on-column DNase digestion was used. RNA was converted to cDNA using SuperScript

First-Strand Synthesis System according to the manufacturer's protocol with random primers (Invitrogen). Relative real-time PCR was then performed using an ABI 7900HT sequence detection system using SYBR GREEN PCR master mix (Applied Biosystems) as follows: stage 1: 50°C for 2 minutes; 95°C for 10 minutes; stage 2 (40-45 cycles): 95°C for 15 seconds; 60°C for 1 minute; 72°C at 20 seconds; stage 3 (dissociation curve): 95°C for 15 seconds; 60°C for 15 seconds; 95°C for 15 seconds. Polymerase chain reaction data were analyzed using Primer Express Software v2.0 (Applied Biosystems). Target mRNA levels were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. The following primer pairs were used:

LAMA3
forward: GACACCAATCTCACAACTCTCCG
reverse: ATGGGGACAGCAACCTTACTGG
LAMA4
forward: GCCGCTTGGTTTACATGTTT
reverse: AATGGGACCCTTGATTTTCC
LAMB1
forward: AAGGATTCCAACCAGCAGCC
reverse: TCATCGGTGTTTTCACAACGC
LAMB2
forward: CCCTGAGCCTGACAGACATAAATG
reverse: TGCTGAGGATGCTACCACCTTC
LAMB3
forward: TCAGAGGAAGAGGGAGCAGTTTG
reverse: GGTCAGGCAACGAAGACATCTC
LAMC1
forward: GAATCATCTAATCCTCGGGGTTG
reverse: TCAAGCACAAGGTCTTCGGCAG
LAMC2
forward: CAGGAGATTGTTATTCAGGGG
reverse: TGGGGTCCACATTGTTGTTGC
GAPDH
forward: GAAGGTGAAGGTCGGAGTC
reverse: GAAGATGGTGATGGGATTTC

Western Immunoblot Analysis

Total cell lysates were prepared by addition of cold lysis buffer (30 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% Triton X-100) containing protease and phosphatase inhibitors (Protease and Phosphatase Inhibitor Cocktail II; Sigma, St. Louis, MO) to monolayer cultures. Conditioned medium was concentrated using Centricon YM-10 columns (Millipore, Billerica, MA). For both lysates and media, total protein concentration was determined with the BCA protein assay kit (Pierce Biological). Reducing sample buffer (Pierce Biological) was added to 25 μg of each sample. Samples were boiled for 5 minutes before running on 5% SDS–PAGE gels. Proteins were then transferred to nitrocellulose in buffer containing 15 mM Tris base, 120 mM glycine, and 5% methanol. Membranes were blocked with Tris-buffered saline (TBS is 20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl) plus 5% nonfat milk. Blots were incubated overnight at 4°C with appropriate antibodies in TBS/0.1% Tween 20/5% nonfat milk. Tris-buffered saline/0.3% Tween 20 was used for all washes. Blots were incubated for 1 hour at room temperature in horseradish peroxidase–linked secondary antibodies, diluted in TBS/0.1% Tween 20/ 5% nonfat milk. Bands were detected using enhanced chemiluminescence reagents (ECL system) according to the manufacturer's protocol.

Immunoprecipitation

Serum-free medium was collected from cells after 24 hours. Five hundred microliters of medium was precleared with 1 μg of appropriate control IgG plus 20 μl of Protein A/G agarose beads (Pierce Biological) for 30 minutes at 4°C. The supernatant was transferred to a new tube and brought up to 1 ml with RPMI T&S. We added 2 μg of either LMα3 or LMγ2 chain antibody to each sample and incubated on a rotating carousel overnight at 4°C. After incubation, we washed the beads three times with 1× PBS and resuspended them in Laemmli sample buffer with β-mercaptoethanol. Samples were boiled for 5 minutes and then loaded onto 5% SDS-PAGE gels (see Western Immunoblot Analysis subsection).

Immunofluorescent Staining

Cells were plated onto sterile round microscope slides in 24-well plates and grown to 70% confluence. After washing with PBS, cells were fixed with a cold methanol/acetone mixture (1:1) for 1 minute, followed by two washes with PBS. Cells were blocked with heat-denatured BSA (0.2%) for 1 hour at room temperature. Primary antibodies [LMα4 (H-194), LMβ2 (H-300), LMα3 (D2-1 or C2-5), LMβ3 (A2′-2), LMγ2 (B46), LMβ1γ1 (R5922), and fibronectin (R790)] were diluted in heat-denatured BSA and incubated at room temperature for up to 3 hours. After PBS washes, fluorescent-conjugated secondary antibodies (anti–rat 488, anti–mouse 488, and anti–rabbit 568) were added for 30 minutes at room temperature. After PBS washes, cells were fixed again with 2% formaldehyde for 10 minutes at room temperature. Cells were viewed and images acquired using a Zeiss fluorescence microscope with a Photometric SenSys cooled CCD digital camera (Roper Industries, Trenton, NJ). Images were analyzed with MetaMorph (Universal Imaging, Downington, PA) and ImageJ (rsbweb.nih.gov/ij/) software.

Cell Proliferation Assays

The rate of cellular proliferation in culture was measured by a colorimetric MTS assay, using the Cell Titer 96 AQueous kit from Promega. M12pc, M12α4, M12β2, M12α4β2, and M12mac25 cells were seeded in 96-well plates at 5000 cells per well in complete RPMI medium. Twenty-four hours later, medium was switched to RPMI minus growth factors and serum. After adding the tetrazolium salt/dye solution for the MTS assays, plates were incubated at 37°C for 2 hours. One 96-well plate was read per day for 5 days. Quantitation was accomplished by reading absorbance at 490 nm; day 1 measurements were used as a baseline. To validate MTS results by direct measurement of cell number, cells were plated in 24-well plates (10^4 cells per well) and grown for 96 hours as in the MTS assay; cell counts were performed every 24 hours using a hemocytometer, again with day 1 used as a baseline (data not shown). Both assays were repeated six times; results shown are the average of the six experiments.

Wounding Assays

Wounding assays were used to assess migration of the cells. M12pc, M12α4, M12β2, M12α4β2, and M12mac25 cells were plated in six-well plates with complete RPMI medium and grown to confluence. After rinsing the cells twice with PBS, the cell layer was scratched with a 10-μl pipette tip, and RPMI T&S medium was added. Wound width was measured at 0, 1, 3, 6, 9, 12, and 24 hours after wounding. Nine hours after wounding yielded the most significant differences in wound closure among the cell lines.

Wounding assays were repeated three separate times; results shown are the average of the three experiments.

Tumor Formation In Vivo

Groups of 10 nude athymic male mice were injected subcutaneously with M12pc, M12α4, M12β2, M12α4β2, or M12mac25 cells (10^6 cells per mouse). Mice were maintained on a laboratory diet *ad libitum* and were monitored weekly for tumor formation and weight gain/loss for a duration of 6 weeks. If tumors were present, tumor volume was calculated using the formula: $(l \times w^2)/2$, where l is length and w is width of tumor. For comparing final tumor volumes at 6 weeks, statistical analyses using analysis of variance followed by Fisher's protected least significant difference (Fisher's PLSD) were performed. After 6 weeks, the mice were euthanized, and their tumors were removed and measured.

Tumor Analyses

Tumors were divided into thirds and treated as follows: (1) fixed in formalin, 1 hour, (2) frozen in optimal cutting temperature (OCT), or (3) digested with 0.1% collagenase (type I) and 50 μg/ml DNase (Worthington Biochemical Corp., Lakewood, NJ). Digested tumor cells were plated in RPMI complete medium plus selective antibiotics at 5% CO_2, 37°C. Cell lysates, media, and RNA were prepared and analyzed by Western blot analysis or real-time PCR to confirm retention of LM expression. Fixed and frozen tumor sections were sliced (5 μm thick for formalin-fixed and 10 μm thick for frozen) on a cryostat and mounted on slides for immunohistochemistry. The presence of tumor vasculature was assessed on frozen tissue using a rat anti–mouse endothelial cell antigen (MECA; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). We used Masson's Trichrome staining on paraffin-embedded tumors to assess the amount of ECM, specifically collagen, present.

Results

Altered Expression of LM Chains in Senescent Prostate Cancer Cells

As mentioned earlier, senescent M12 and LNCaP prostate cancer cells had increased LMα4 and β2 chain transcripts on cDNA microarrays. To confirm increased expression in the senescent M12mac25 prostate cancer cell line, we used real-time PCR and Western immunoblot analysis. mRNA and protein levels of the LMα4 and β2 chains were increased in the M12mac25 cells compared to the M12 empty vector (M12pc) cells (Figure 1, *A* and *B*). Because LM332 (α3β3γ2, formerly LM-5) is the predominant LM in prostate, we evaluated the levels of the component chains of LM332 in the M12 prostate cancer lines and compared them to levels found in PECs. We found that mRNA and protein levels of all three chains (α3, β3, and γ2) were decreased in both the nonsenescent M12pc and senescent M12mac25 cells compared to the PECs (Figure 1, *A* and *B*), mirroring the decrease in LM332 that has been reported to occur *in vivo* during prostate cancer progression [28].

Construction of M12 LM α4, β2, and α4β2 Chain-Overexpressing Cells

To examine the role of senescence-induced LM chain expression in prostate cancer tumorigenesis, we created M12 prostate cancer cell lines stably overexpressing the LM α4 or β2 chains or both the α4 and β2 chains. Transfections were repeated three to four times with each construct to ensure consistency of characteristics. All of the transfected cells stably overexpressed both mRNA (Figure 2*A*) and protein (Figure 2*B*) of the selected LM chains. Like M12pc cells, high-expressing M12α4 populations were cuboidal (Figure 3), whereas the high-expressing M12β2 and M12α4β2 populations were a mix of cuboidal and elongated cells (Figure 3). The appearance of cell populations with an elongated morphology was intriguing because we have previously shown that M12 cells overexpressing the senescence-associated gene *mac25* or the chondrogenesis-associated transcription factor SOX9 (which is up-regulated on arrays of senescent epithelial cells) [21,29] also displayed an elongated morphology and overexpressed both the LMα4 and β2 chain mRNA and protein [19,20,30].

Immunofluorescent Staining

Whereas immunofluorescent staining for various LM chains demonstrated patterns similar to those found in the Westerns, it also provided the opportunity to determine whether the cells deposited the various LM chains into the ECM. Sigle et al. [31] demonstrated that cell lines

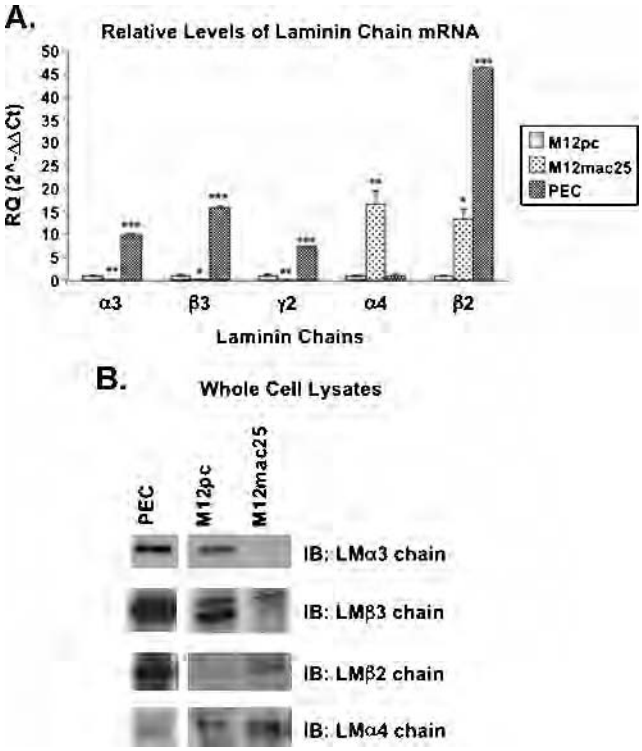

Figure 1. mRNA and protein levels for the various LM chains in prostate cells. (A) Real-time PCR for LM chains. RNA from PEC, M12 prostate cancer cells, and M12mac25 cells was converted to cDNA then amplified using primers for the various LM chains. No significant differences among cell lines were detected for the LMβ1 or γ1 chains. $^*P < .005$, $^{**}P < .001$, $^{***}P < .0001$ compared to M12 levels. (B) Western immunoblots against various LM chains. Whole cell lysates, which include cytoplasmic and ECM-deposited LM chains, were run on reducing SDS-PAGE gels, transferred to nitrocellulose, and blotted with antibodies against various LM chains. No differences in protein expression were seen for the LMβ1 and γ1 chains.

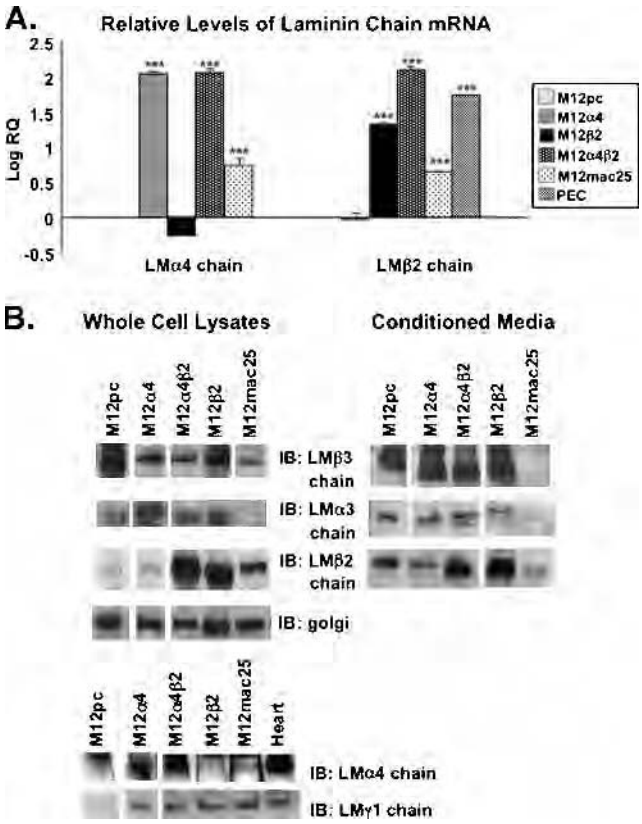

Figure 2. Relative mRNA and protein levels of LM chains in the various M12 LM cells compared to levels in the M12 control cells. (A) Laminin chain cycle numbers were normalized against GAPDH cycle numbers (ΔC_t). M12 normalized cycle numbers were subtracted from the M12 LM cells (M12α4, M12α4β2, and M12β2) and M12mac25 cell numbers to yield $\Delta\Delta C_t$ values. The following formula was used to derive relative quantitation values: RQ = $2^{-\Delta\Delta Ct}$. Primary prostate epithelial cell levels are included to show relative physiological levels of the LM α4 and β2 chains in primary prostate cells compared to the transfected levels in the various M12 LM cells. mRNA levels of the introduced laminin chains were significantly higher for the M12 LM cells compared to the control M12 cell ($P < .0001$). As with the M12 control cells, LM332 levels in the M12 LM cells remained significantly lower than in the PECs ($P < .001$; data not shown). (B) Western immunoblots for various LM chains. Either whole cell lysates or concentrated conditioned media were run on reducing SDS-PAGE gels. Heart, which expresses very high levels of the LMα4 chain, was used as a positive control. Murine heart was homogenized and lysed with the same lysis buffer as the M12 LM cells.

do not necessarily deposit the LMs they secrete; thus, it was important to determine which LM chains were being incorporated into the ECM. LM332 is classically laid down in a monolayer, which *in vitro* is described as exhibiting a rose petal pattern. Staining for the three LM chains found in LM332 showed some deposition of LM332 in a monolayer for the M12pc and M12 LM cells but not for the M12mac25 cells (Figure 4*B*). In addition, the M12α4β2 cells, and to a lesser degree the M12β2 cells, displayed a three-dimensional fibrillar staining pattern for the three chains comprising LM322 (Figure 4*B*). Staining for the LMβ2 chain demonstrated primarily cytoplasmic staining in the LMβ2-overexpressing cells (M12β2, M12α4β2, and M12mac25 cells), with occasional fibrillar staining (Figure 4, *A* and *B*). When the cells were removed before staining, faint staining for the LMβ2 chain could be detected in the ECM from LMβ2-overexpressing cells but not in ECM from M12pc or M12α4 cells (Figure 4*B*). We were not able to successfully use the commercial LMα4 chain antibody for staining of cells; however, when we removed the cells before staining the matrix, we detected faint staining of the LMα4 chain in the M12α4β2 and M12mac25 matrices (data not shown). The LMβ1 and γ1 chains stained similarly for all the M12 LM cell lines; these chains were located in both the cytoplasm and, to a lesser extent, the ECM (Figure 4, *A* and *B*). The punctate staining pattern seen for the LMα4 and β2 chains in the matrix of the M12mac25 cells was even more pronounced for the LMβ1 and γ1 matrix staining (Figure 4*B*), indicating that these LM chains are deposited in a different manner than LM332 in these prostate cancer cells.

Altered expression of one ECM protein is often associated with changes in other ECM proteins. Expression of fibronectin (FN), a fibrillar ECM protein, has been shown to alter during senescence, aging, and cancer progression [29,32–34]. We found that the cell populations overexpressing the LMβ2 chain and displaying an elongated morphology had increased amounts of fibrillar staining for FN compared to the M12pc and M12α4 cells (Figure 4*A*). Staining of FN after removal of the cells yielded a pronounced punctate staining pattern for all the cells (Figure 4*B*).

Positive cytoplasmic staining of the various LMs occurred equally well in both cuboidal and elongated cells; however, the amount of three-dimensional matrix staining present correlated with the predominance of an elongated morphology. The cell populations containing the largest number of elongated cells were also the ones overexpressing the LMβ2 chain, suggesting that the higher protein levels of the LMβ2 chain seen in the M12β2 and the M12α4β2 cells may lead to altered three-dimensional deposition of matrix proteins, including LMs and fibronectin.

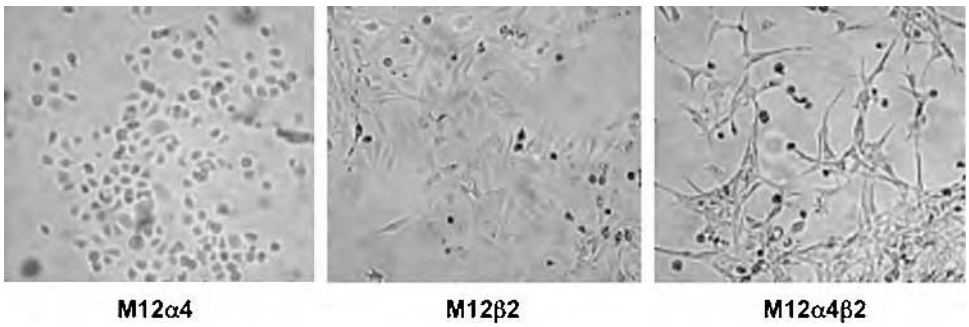

Figure 3. Morphology of transfected cells. M12α4 cells were similar in appearance to the M12 control cells. The M12β2 and M12α4β2 cells were a mix of cuboidal and elongated cells.

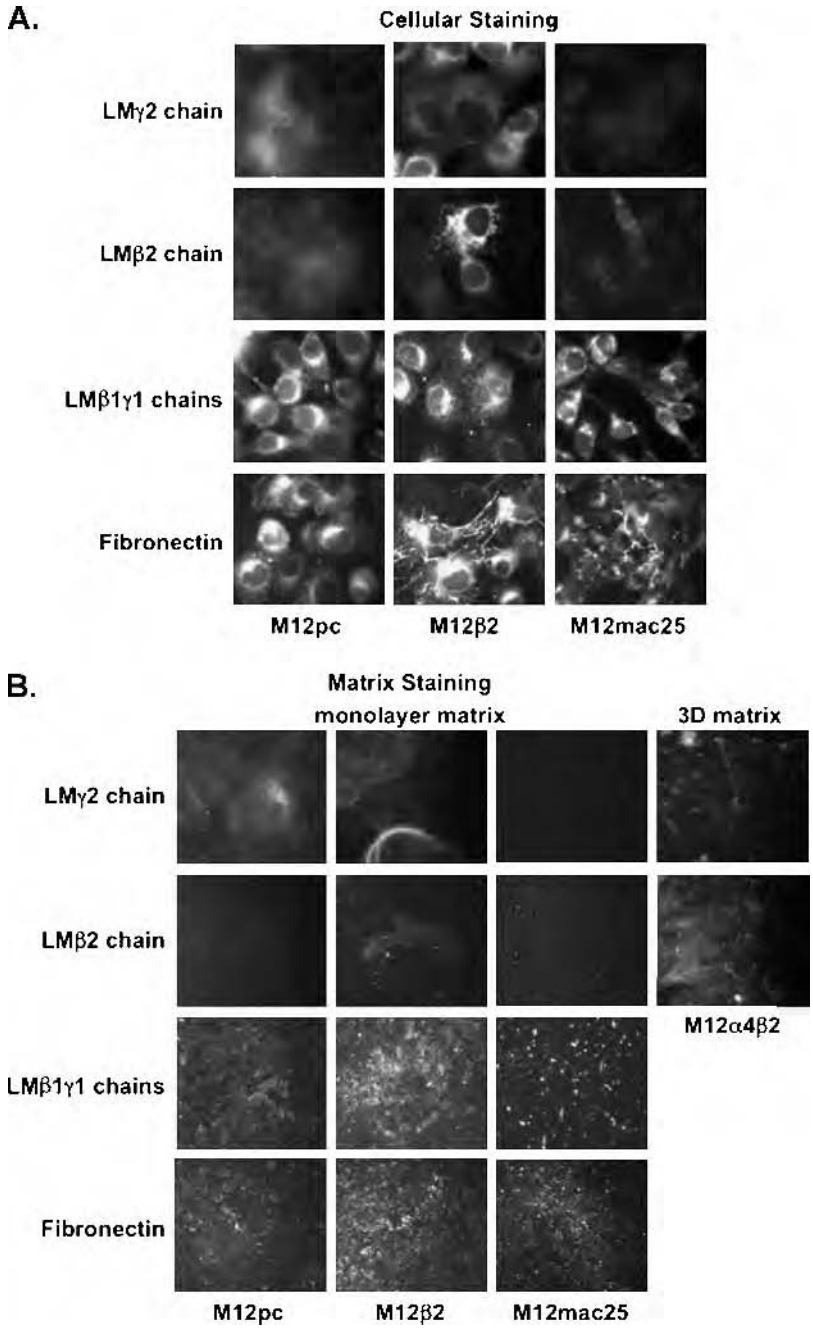

Figure 4. Immunofluorescent staining of various LM chains and fibronectin in the M12 LM cells. (A) Cells were fixed and stained for various LM chains or fibronectin. All three LM332 chains stained similarly; LMγ2 chain staining is shown. (B) Staining of matrix after cells were removed with 0.5% Triton X and 20 mM ammonium acetate. Removal of cells leaves a monolayer matrix and occasionally a three-dimensional matrix, as was the case for the M12α4β2 cells. Removal of cells provided a clearer picture as to what LM chains were being deposited in the ECM. Note the punctate staining pattern of fibronectin is similar to the pattern for the LMβ1γ1 and β2 matrix staining.

Association of the LMβ2 Chain with LM332

Dual staining with the LMβ2 chain antibody and LM332 antibodies demonstrated colocalization of these LM chains in the three-dimensional fibrillar matrix as well as in the monolayer matrix (Figure 5*A*). Likewise, immunoprecipitation of conditioned culture medium showed association of the LMα3 chain with the β3 and β2 chains in cells overexpressing the LMβ2 chain; M12mac25 cells were the exception because they produce very little of the LMα3 chain but do express the LMβ2 chain (Figure 5*B*). Immunoprecipitation with the LMγ2 chain also demonstrated an association with the LMβ2 chain (Figure 5*C*).

In Vitro *Cellular Proliferation and Migration*

To determine whether alterations in LMs affected proliferation, we performed MTS assays. Five days after plating, M12β2 cells had the highest proliferation rates followed by M12α4, M12α4β2, and M12mac25 cells (Figure 6*A*). Next, we used wounding assays to assess if altered LM production influenced cell migration. After wounding,

cells redeposit matrix on which they migrate. All of the cells, except M12mac25, deposit similar amounts of LM332 but differ in their production of the LMα4 and β2 chains. Cells were wounded and then exposed to RPMI medium minus growth factors and serum. Wound closure was measured at early time points, such as 9 hours, which were representative of migration rather than proliferation. The M12α4 and M12β2 cells demonstrated a trend toward increased migration compared to the M12pc cells; further, these cells had significantly increased migration compared to the M12α4β2 and M12mac25 cells ($P < .05$; Figure 6*B*). After 24 hours, all the M12 LM cells had piled up along the original wound, whereas the M12pc cells had simply closed the gap, and the M12mac25 cells had failed to migrate into the wound (Figure 6*C*). Because both the M12α4β2 and M12mac25 cells proliferate poorly, these results suggest that the wound closure and the piling up of cells in the M12α4β2 cultures were caused by the increased migration of these cells.

In Vivo *Tumorigenicity*

To examine *in vivo* tumorigenicity of the LM-overexpressing cells, we injected 10^6 cells subcutaneously from each cell line into groups of 10 male athymic nude mice and monitored tumor growth during a period of 6 weeks. By 6 weeks, 100% of the M12β2, 90% of the M12α4, and 70% of the M12α4β2 mice had tumors. In comparison, 100% of the mice injected with M12pc control cells had tumors and none of the mice injected with M12mac25 had tumors. After 6 weeks, the average tumor volume was significantly higher ($P < .05$) for the M12β2 group compared to the M12α4β2 group (Figure 7). Whereas the tumor volumes of the other groups were not significantly different, the trend in tumor volume mirrored the trends seen for *in vitro* proliferation and migration.

To determine whether LM overexpression was maintained in the tumors, we digested a portion of each tumor with collagenase. Antibiotic-resistant cells were regrown in tissue culture, and total RNA, whole cell

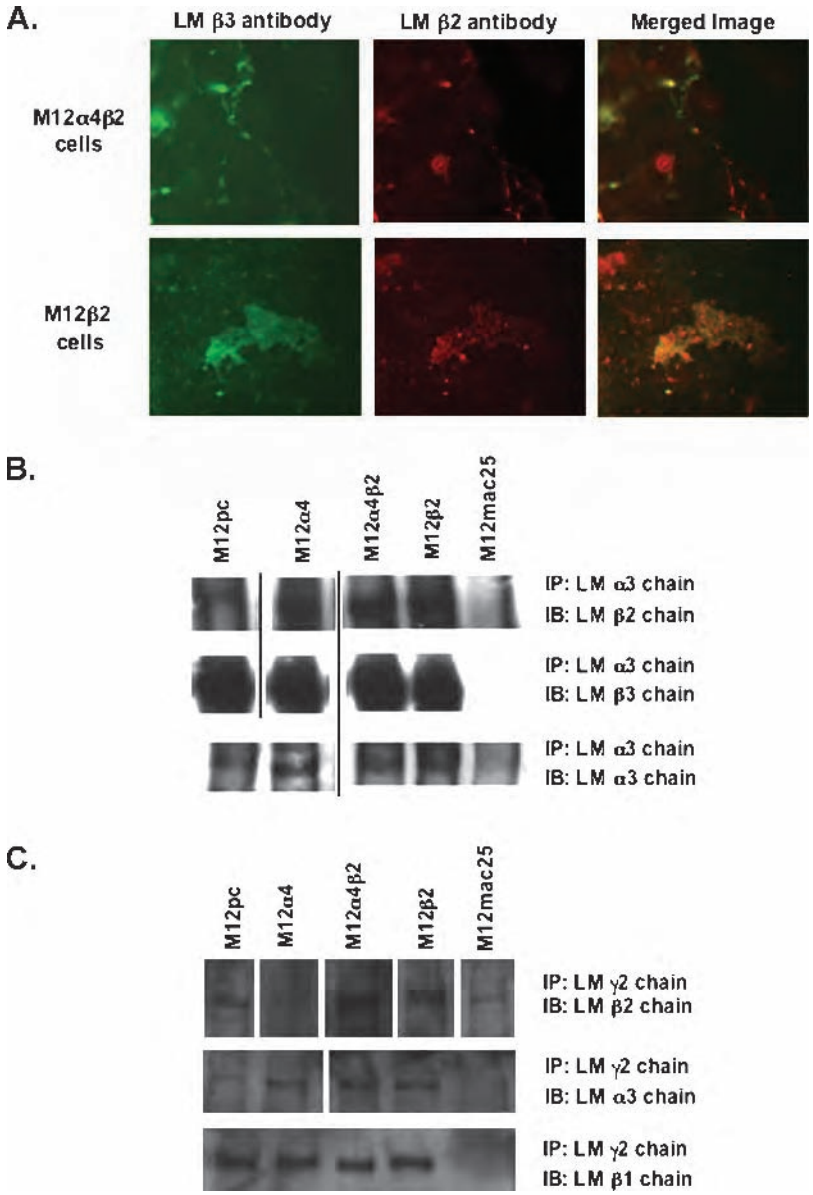

Figure 5. Colocalization of LMβ2 chain with LM332 chains. (A) Dual staining of matrix revealed colocalization of the LMβ2 chain with chains found in LM332 in both the three-dimensional matrix and the matrix monolayer. (B and C) Immunoprecipitations (IPs) using the LMα3 and LMγ2 antibodies followed by blotting with the LM β2 chain antibody demonstrated the presence of this chain in both LMα3 and LMγ2 IPs.

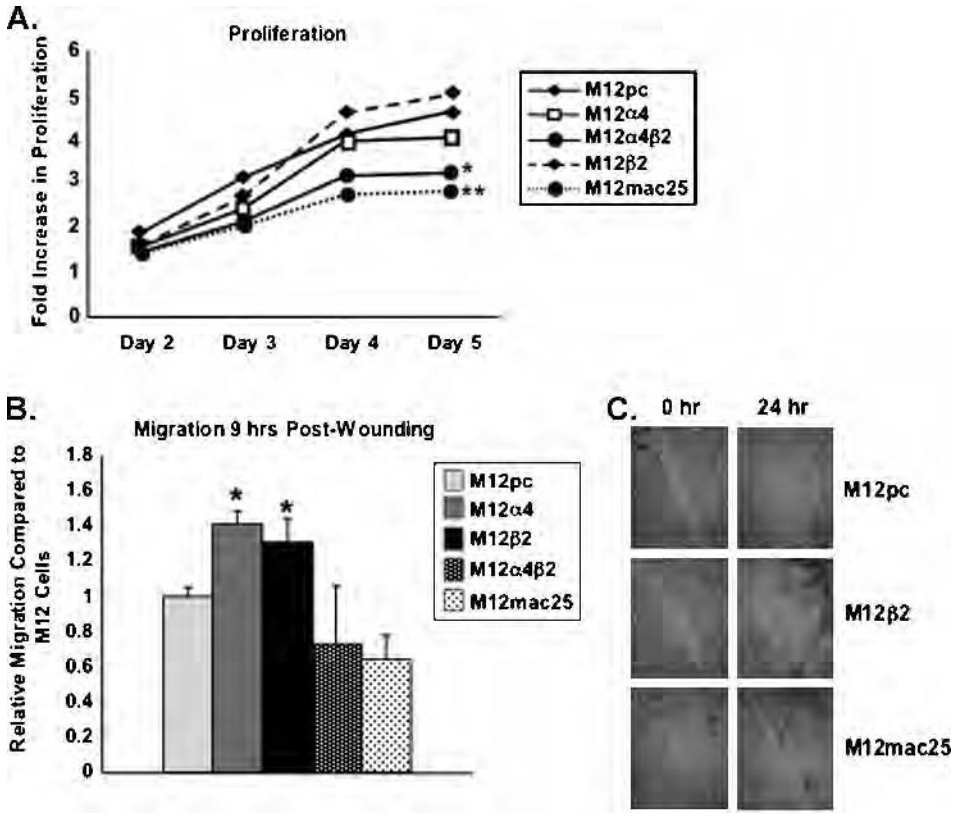

Figure 6. *In vitro* cellular proliferation and migration of the M12 LM cells compared to the M12 control cells. (A) Cellular proliferation was measured with the MTS assay, which correlated to cell number. *$P < .01$, **$P < .001$ compared to M12β2 cells. (B) To determine whether alterations to LM chain expression modulated the ability of the cells to migrate, we performed *in vitro* wounding assays as described in the Methods section. The greatest differences in migration were seen at 9 hours after wounding. (C) Twenty-four hours after wounding, all the cells except for the M12mac25 line had closed the wound. M12 LM cells piled up along the wound site, whereas the site of the original wound could not be detected in the M12 control cells.

lysates, and conditioned media were all collected and analyzed for LM expression. mRNA levels for the LMα4 and β2 chains decreased significantly ($P < .05$) in the tumors compared to preinjection levels; however, the mRNA levels remained significantly higher than the LMα4 and β2 chain levels seen in the M12pc cells ($P < .0001$) and M12pc tumors ($P < .0001$; Figure 8*A*). Western immunoblots demonstrated maintenance of increased protein levels of the introduced LM chains (Figure 8*B*).

In various cancers, alterations in LMs have been associated with changes in expression of other matrix proteins and angiogenesis [22]. We examined the tumors for overall matrix composition; histologic comparison of tumors demonstrated significantly more collagen deposition in the matrix for the M12 LM-overexpressing lines compared to the M12pc tumors ($P < .05$; Figure 9). Because alterations in both collagen and LM expression, especially an increase in LM411 (α4β1γ1), occur during tumor angiogenesis, we stained tumors against MECA. Because M12 LM cells are human, the MECA staining detects the host-derived tumor blood vessels. M12α4 tumors had the highest staining intensity for MECA followed by the M12β2, M12α4β2, and M12pc tumors (Figure 10*B*).

Discussion

Laminins are expressed in both normal and malignant prostate tissue, but different isoforms predominate in each case. In nonmalignant prostate ECM, LM332 is predominant [26,28,35]; the high mRNA and protein levels of the LM α3, β3, and γ2 chains observed in the PECs are consistent with previous data. LM332 has been shown to be necessary for epithelial cell polarization during development of a normal basal epithelial cell layer [36,37]. Without LM332, the basal cell layer fails to develop, and the epithelial cell loses the cell-cell contact protein E-cadherin, as well as other structurally significant molecules, and is prone to phenotypic deregulation and transformation [26]. LM332 is lost in progression to prostate cancer, and further alterations, including cleavage of the α3 and β3 chains, occur as the cancer becomes invasive or metastatic; these alterations and the loss of normal LM332 may contribute to formation of a structurally weaker ECM more conducive to metastases [26,28,38]. The mechanisms responsible for loss of LM332 are not well understood, although aberrant methylation of promoter regions could lead to silencing of LM332 genes [39]. Various prostate cancer cell lines, including the LNCaP line, have already been shown to mirror the *in vivo* loss of LM332 [40]; we demonstrate here that another prostate cancer cell line, M12, has significantly decreased its production of LM332.

We found that nonsenescent PECs expressed abundant levels of the LMβ2 chain but did not express the LMα4 chain. Our senescent M12mac25 cells showed partially restored LMβ2 chain mRNA and protein levels compared to PECs and demonstrated increased levels of LMα4 chain mRNA and protein compared to both PECs and M12 cells. Other laboratories have reported an increase in the LMα4 chain transcript in senescent primary prostate fibroblasts [6] and senescent prostate epithelial cells [6,41]. Our results suggest that prostate cancer cells induced to undergo senescence alter their LM production as well.

Several studies have shown that LM expression is altered during tumor progression [22,26]. In glial tumors, abnormalities in matrix production develop in glial cells, adjacent stroma, and endothelial cells. As in prostate cancer, there is loss of LM332 to a degree depending on the depth within the tumor and the grade of tumor [25,42,43]. Gliomas also exhibit elevated levels of the LMα4 chain, and during glioblastoma progression, endothelial cells cease synthesis of LM421 (α4β2γ1) in favor of LM411 [25,42]. This conversion can be induced by coculture of glioblastoma cells and endothelial cells, and the malignant phenotype can be reversed by reexpression of LM421 [43]. A similar effect may occur in the prostate where senescent stromal cells have been postulated to influence the cancer phenotype of prostate epithelium. Prostate cancer cells can sometimes integrate with tumor vessels and coexpress vascular antigens, a phenomenon termed *vasculogenic mimicry*, which facilitates tumor progression [44,45]. These observations suggest that selective expression of LM411 and 421 influences the proliferative or differentiated phenotype and that aberrant expression of LM411 by senescent or tumor cells can influence the angiogenic potential of adjacent endothelial cells, which in turn facilitates tumor growth and metastasis.

Because LMs are known ligands for various cell surface receptors, they are important biologically as mediators of cellular behaviors such as proliferation, migration, and tumorigenicity. However, there is a paucity of studies examining the direct role of LMs in prostate cancer or senescence. Most studies on alterations in LM composition during cancer progression focus on immunohistochemical changes in prostate tissue samples [26,28,35]. The few functional studies on LMs have concentrated on LM511 (α5β1γ1) and its cleavage products and on their role in increased migration of prostate cancer cells [46,47]. One study has been reported in which the LMβ3 chain was transfected into the LNCaP prostate cancer cell line. Although punctate deposition of the LMβ3 chain occurred along cell surfaces, the authors were unable to detect either secretion or the classic monolayer deposition of the LM332 trimer into the matrix. However, the reexpression of the LMβ3 chain still resulted in increased tumor formation *in vivo* and altered expression of genes involved in various growth signaling pathways on cDNA arrays [40], which suggests that individual LM chains may have unexplored biological functions.

Our LM transfected cells, however, secreted and deposited the introduced LM chains. Furthermore, we found that introduction of the LMβ2 chain was associated with an alteration in deposition of the endogenous LM332. Compared to the M12pc empty vector cells and the M12α4 cells, staining against the LM332 chains in the M12β2 and M12α4β2 cells showed both a classic monolayer deposition and a fibrillar three-dimensional staining pattern. Whereas primary epithelial cells normally deposit LM332 in a monolayer

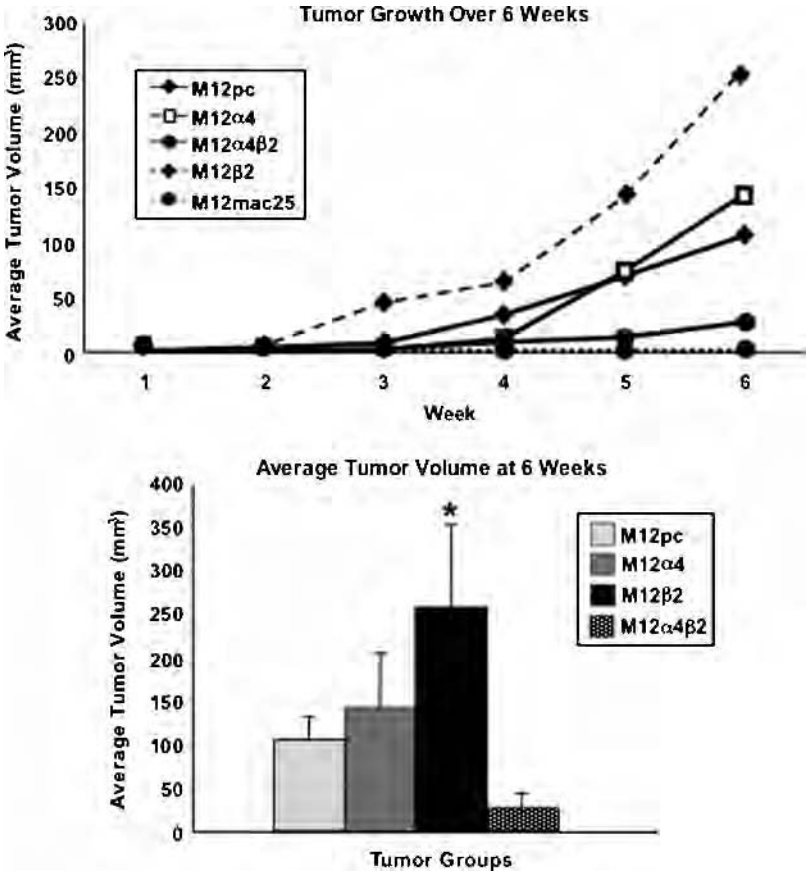

Figure 7. *In vivo* tumorigenicity of the various M12 LM cells compared to M12 control cells. Groups of 10 male athymic nude mice were subcutaneously injected with 1×10^6 cells and followed for 6 weeks. Tumor volume was calculated using the following formula: $(l \times w^2)/2$. Mice injected with M12β2 cells displayed the greatest tumor growth, whereas mice injected with M12α4β2 displayed the slowest tumor growth. $^*P < .01$ compared to M12β2 tumors. M12mac25 cells did not form tumors *in vivo*.

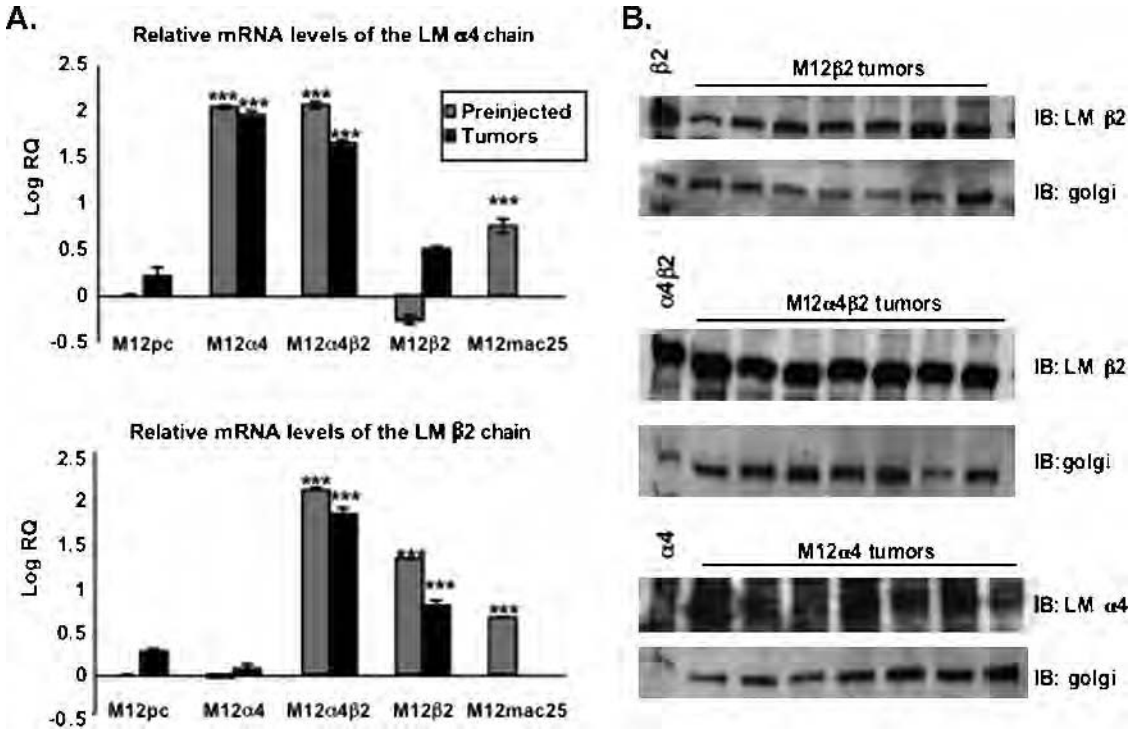

Figure 8. Relative LMα4 and β2 expression in *in vivo* tumors. A portion of each tumor was digested with collagenase, then the antibiotic-resistant cells were regrown *in vitro* and total RNA and whole cell lysates were collected. (A) RNA was converted to cDNA, and real-time PCR was run using primers against the LMα4 and β2 chains. Whereas the levels of introduced LM chain mRNA decreased compared to preinjected levels, the tumor levels remained significantly higher than the LMα4 and β2 chain levels seen in the M12 control cells ($P <$ .0001) and M12 tumors ($P <$ .0001). (B) Western immunoblots against the LMβ2 and α4 chains. Lysates were run on reducing SDS-PAGE gels. As shown here, the introduced LM chains were still highly expressed by the tumor cells.

in vitro, three-dimensional deposition of LM332 can occur *in vitro* when these cells are cocultured with fibroblasts that secrete large amounts of fibronectin. The epithelial cells deposit LM332 along the fibronectin fibers, separating themselves from the fibroblasts in discrete "nests" [31]. Immunohistochemical studies on various cancers, including prostate, demonstrate that primary tumors often form their own basement membrane, separating themselves from the surrounding stroma [4,22,26,48].

Our observation that the introduction of the LMα4 and β2 chains altered the deposition of matrix proteins prompted our exploration of the effects of altered LM expression on cell behavior. We found that *in vitro* proliferation and migration were, in general, enhanced by increased expression of either the LMα4 or β2 chains but decreased if both chains were highly expressed. *In vivo* tumor formation followed the same trend. Mouse endothelial cell antigen staining of these tumors demonstrated increased tumor vasculature in the M12α4 tumors compared to the M12β2 and M12α4β2 tumors. These results agree with immunohistochemical studies on breast cancer and glioblastoma samples, which demonstrated that high levels of LMα4 protein expression were associated with tumor vasculature and increased tumorigenicity, whereas expression of both the LMα4 and β2 chains was associated with decreased tumorigenicity and normal vasculature [24,25,49].

Only a few studies examining the LMβ2 chain in cancer have been reported. Immunohistochemical studies on LMs suggest increased staining for the LMβ2 chain in prostate cancer tissue samples compared to normal prostate [48] and increased LMβ2 staining in ovarian tumor basement membranes, especially in lower-grade cancers

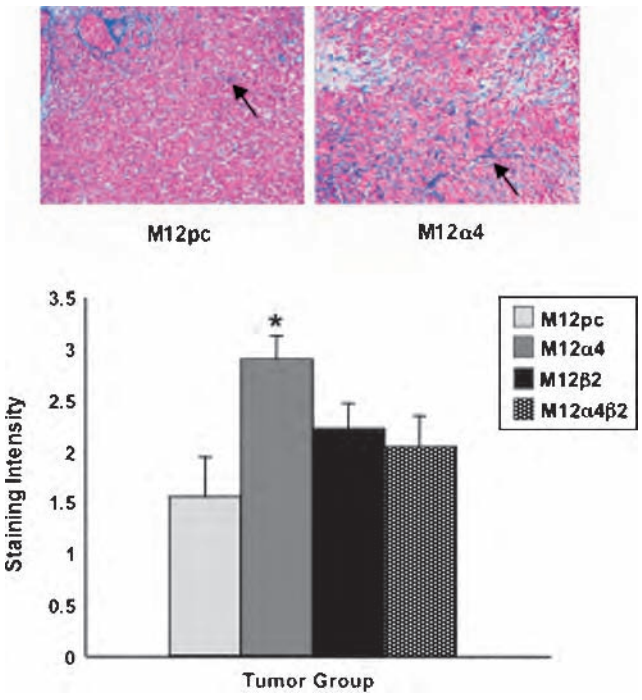

Figure 9. Collagen staining of *in vivo* tumors. Masson's Trichrome staining, which stains primarily the collagen component of the ECM, was performed on fixed tumor sections. Staining intensity was scored for each tumor, and the average intensity was calculated per group. The amount of collagen present was significantly higher for the M12α4 tumors ($P <$ .05) compared to the M12 tumors.

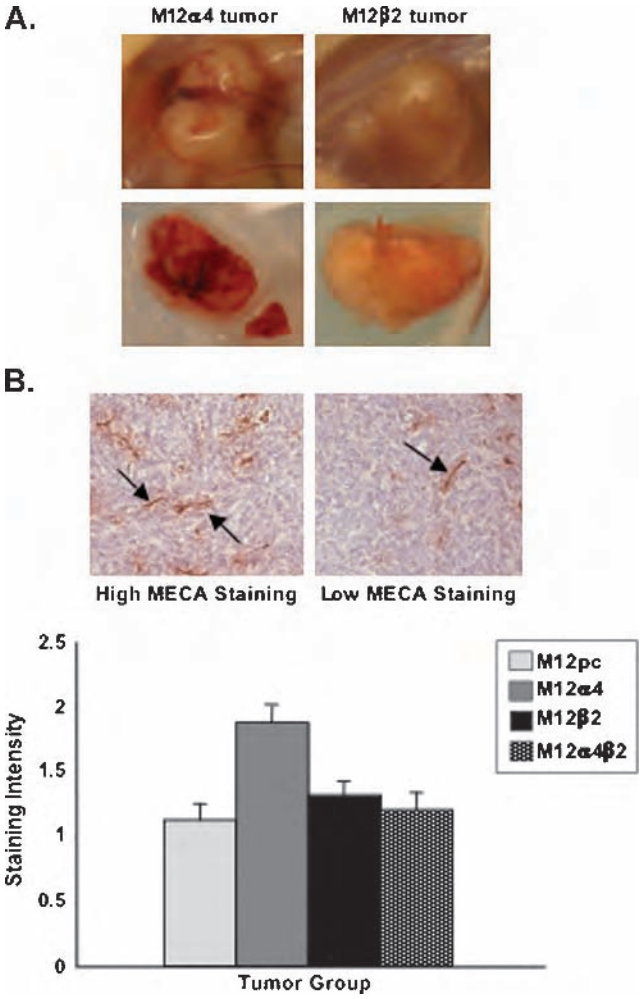

Figure 10. Differences in tumor angiogenesis. (A) On a macroscopic level, M12α4 tumors had an enhanced blood supply compared to M12β2, M12α4β2, and M12pc tumors. (B) To assess the presence of host-derived vessels microscopically in the tumors, staining against MECA was performed on frozen tumor sections. M12α4 tumors had more vessels than M12pc, M12β2, or M12α4β2 tumors.

[50,51]. In addition, there is one report in the literature regarding hypovascular, mega-adenomas in pituitary cancers. Using an *in vitro* model of hypoxia in pituitary tumors, Bao et al. [52] demonstrated a correlation between increased LMβ2 chain protein (but not the other LM chains) and decreased oxygen concentration. Further, immunohistochemical analysis of pituitary adenoma sections showed a significant correlation between staining intensity for the LMβ2 chain and increased size of the tumor. It would be of interest to see what role hypoxia plays in the relatively hypovascular M12β2 tumors.

The previously mentioned studies, however, did not determine the identity of the mature β2-containing LM trimer involved in these ECM-rich cancers. We have presented data suggesting that the LMβ2 chain is associated with the LMα3 chain in the M12 prostate cells overexpressing the LMβ2 chain. Whether this is a direct association resulting in a LM321 trimer or simply a β2-containing LM complexing with LM332 is not known because immunoprecipitations with both the LMα3 and γ2 chains pulled down the LMβ2 chain, and immunofluorescent staining demonstrated costaining of the LMβ2 and LMβ3 chains as well. Champliaud et al. [53] suggest that the LMβ2 chain can directly bind the LMα3 chain to form a LM321 trimer. Further, they argue that LM332, LM321, and LM311 form a complex in normal epithelial basement membranes. Therefore, a complex of LM332, 311, and 321 is a possibility in the LMβ2-overexpressing cells, although the formation of such a complex has not been examined in cancer cells.

Expression of the LMβ2 chain alone has a tumor promoting function as opposed to the tumor suppressive role seen with high protein levels of the LMα4 and β2 chains together. The mechanisms behind this dichotomy in function for the LM β2 chain remain to be elucidated, but this observation supports the idea that both tumor-promoting and tumor-inhibiting factors secreted by senescent cells combine or interact to influence cellular behavior. Identification of the factors secreted by senescent cancer cells and their effects on the regression or progression of the remaining tumor will have important implications for future cancer modalities.

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